PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61L 29/00, 31/00

A2

(11) International Publication Number: WO 95/13103

(43) International Publication Date: 18 May 1995 (18.05.95)

(21) International Application Number: PCT/US94/12894

(22) International Filing Date: 9 November 1994 (09.11.94)

(30) Priority Data:
08/151,134 12 November 1993 (12.11.93) US

(71) Applicant: DESMOS, INC. [US/US]; Building 2, Room 214, 3550 General Atomics Court, San Diego, CA 92121 (US).

(72) Inventors: QUARANTA, Vito; 8861 Nottingham Place, La Jolla, CA 92037 (US). HORMIA, Marketta; 3665 Morlan, San Diego, CA 92117 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson & Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: SOLUBLE FACTOR STIMULATION OF ATTACHMENT AND HEMIDESMOSOME ASSEMBLY IN EPITHELIAL CELLS

(57) Abstract

A method for growing epithelial cells *in vitro* using soluble proteins secreted by 804G rat bladder carcinoma cells. These proteins are able to stimulate cell attachment and hemidesmosome formation in cells grown in contact with the proteins. The purification of these proteins from 804G culture supernatant is greatly facilitated by culturing the cells under low serum conditions. Trans-epithelial appliances coated with the soluble proteins and the use of the soluble proteins in the maintenance of human tissue *ex vivo* are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Soluble Factor Stimulation of Attachment and Hemidesmosome Assembly in Epithelial Cells

Background of the Invention

When organs of the body are formed, they develop in neatly organized arrays. Often, cell groups of one kind are separated from cells of another kind by flat strips of connective tissue called basement membranes. In skin, for instance, the superficial layer of epidermal cells adheres to the underlying basement membrane. This skin basement membrane acts as a barrier between the epidermal cells on the outside, and the dermal cells underneath. A similar arrangement of cells occurs in the lining of the gut.

Basement membranes have been implicated in the growth, attachment, migration, repair, and differentiation of their overlying cell populations. Three layers have been defined in basement membranes: a) the Lamina lucida, an electron microscope-clear region that resides in close approximation to the overlying cells; b) the lamina densa, an electron dense region of 20-300 nm in width; and c) the sublamina densa that contains anchoring fibrils, microfibrillar bundles and collagen fibers.

Many different types of compounds have now been localized to the basement membrane. Some of these compounds are laminin, collagen IV and heparin sulfate proteoglycans (Verrando et al. *Exp. Cell Res.* (1987); 170: 116-128). In addition, specific basement membranes have been found to possess other compounds, such as nidogen and entactin.

The principal cell adhesion receptor that epidermal cells use to attach to the basement membrane is called $\alpha_6\beta_4$. This transmembrane receptor is formed by a combination of two protein moieties α_6 and β_4. The α_6 and β_4 proteins are derived from different genes that have

been found to be part of the integrin family.

Integrins are a versatile family of cell adhesion receptors. So far, approximately twenty members of the integrin family have been discovered. These molecules are involved in many types of cell adhesion phenomena in the body. Integrins are signalling molecules that can translate environmental cues into cellular instructions. Further, integrins can also transmit signals in the reverse direction, from the cell interior to the exterior. This has been illustrated in non-adherent cells, such as lymphocytes.

Stimulation of the T-cell antigen receptor, or of the CD3 complex, augments the affinity of certain integrins for their respective ligands. Unfortunately, in adherent cells, changes in the affinities of integrins have been more difficult to demonstrate. However, affinity modulation of one integrin in differentiating epidermal keratinocytes has been described by Adams et al. (*Cell* (1990); **63**: 425-435). For this reason, modifications of cell status initiated by activation or differentiation of other receptors may influence integrin affinity, and ultimately, the adhesive behavior of cells. Further, as a consequence of adhering to a surface, an integrin may actively contribute to modifying cell shape or migration.

Many epithelial cells interact with the underlying extracellular matrix via a junction called the hemidesmosome (Staehelin, (1974) *Structure and Function of Intercellular Junctions*, 191-283). Over the last few years there has been considerable progress in the biochemical characterization of this junction (Schwartz, et al., (1990) *Annu. Rev. Cell Biol.*, **6**:461-491). The hemidesmosome, with its associated structures such as intermediate filaments and anchoring fibrils, forms an adhesion complex. Disruptions of the epithelial-connective tissue interaction is often accompanied by a

disruption of the hemidesmosome complex. For example, in certain blistering skin diseases such as junctional epidermolysis bullosa where epithelial cell-connective tissue interactions is abnormal, it has been proposed that there is a biochemical modification in or loss of a basement membrane zone-associated component of the hemidesmosome.

Two high molecular weight intracellular components of the hemidesmosome have been identified and characterized with the aid of antisera from patients suffering from bullous pemphigoid. This autoimmune disease results in a disruption of the interactions between epithelial cells and connective tissue simultaneously with loss of hemidesmosome integrity (Chapman et al. *Br. J. Dermatol* (1990); **123**: 137-144). Accordingly, it was discovered that bullous pemphigoid patients were producing antibodies against hemidesmosome components. Two hemidesmosome related bullous pemphigoid (BP) antigens have been previously described (Klatte, et al., 1989).

One BP antigen is a 230 kD polypeptide that may act as an anchor for cytoskeleton elements in the hemidesmosomal plaque (Jones and Green, (1991) *Curr. Opin. Cell Biol.*, **3**:127-132). A second BP antigen is a type II membrane protein that possesses a collagen-like extracellular domain (Giudice, et al., (1991) *J. Clin. Invest.*, **87**:734-738; Hopkinson, et al., (1992) *J. Invest. Dermatol.*, **3**:264-270). In addition, it has been demonstrated that the interaction of the hemidesmosome with the underlying connective tissue involves the $\alpha_6\beta_4$ integrin heterodimer (Stepp, et al., (1990) *Proc. Natl. Acad. Sci. USA*, **87**:8970-8974; Jones, et al., (1991) *Cell Regulation*, **2**:427-438; Sonnenberg, et al., (1991) *J. Cell Biol.*, **113**:907-917; Kurpakus, et al., (1991) *J. Cell Biol.*, **115**:1737-1750). The $\alpha_6\beta_4$ heterodimer has been localized to hemidesmosomes along the basal surfaces of

the rat bladder carcinoma cell line 804G (Jones et al. *Cell Regulation* (1991); **2**: 427-438). These results suggested that integrins (e.g. $\alpha_6\beta_4$) may play an important role in the assembly and adhesive functions of hemidesmosomes.

Previous efforts have focused on purifying adhesion-facilitating proteins found in basement membrane. For example, Burgeson, et al., Patent Cooperation Treaty Application Nos. WO92/17498 and WO94/05316, disclose a protein which they call kalinin. Kalinin is said to facilitate cell adhesion to substrates; however, this material is apparently inactive with respect to hemidesmosome formation. See also, Marinkovich, et al., *J. Cell Biol.* (1992); **119**:695-703 (k-laminin); Rouselle, et al., *J. Cell. Biol.* (1991); **114**:567-576 (kalinin); and Marinkovich, et al., *J. Biol. Chem.* (1992); **267**:17900-17906 (kalinin).

Similarly, a basement glycoprotein of about 600 kD comprising polypeptides in the range of 93.5 kD to 150 kD has been identified, and is known as GB3 or nicein. See, e.g., Verrando, et al., *Biochim. Biophys. Acta* (1988); **942**:45-56; and Hsi, et al., *Placenta* (1987); **8**:209-217. None of these materials have been effective in inducing hemidesmosome formation, either *in vitro* or *in vivo*.

When cultured on tissue culture plastic *in vitro*, most epithelial cells do not assemble bona fide hemidesmosomes despite the fact that they appear to express all of the hemidesmosomal plaque and transmembrane components mentioned above. Indeed, it is only recently that cell lines such as 804G were discovered to have the ability to readily assemble hemidesmosomes *in vitro* under regular culture conditions (Riddelle, et al., (1991) *J. Cell Biol.*, **112**:159-168; Hieda, et al., (1992) *J. Cell Biol.*, **116**:1497-1506). Such cells are at last allowing detailed cell and biochemical analysis of the dynamics of hemidesmosome

assembly.

For instance, it has been reported that substratum-associated staining by anti-hemidesmosome antibodies is greatly diminished in 804G cell cultures that enter *in vitro* wound sites (Riddelle et al., *J. Cell Sci.* (1992); 103: 475-490). However, as closure of the wound became complete, anti-hemidesmosome staining along the substratum- attached surface was evident in the cells.

There are, however, many epithelial cells that do not attach to tissue culture dishes in a normal fashion, even after treatment with various growth factors. These cells do not produce normal hemidesmosomes or grow to resemble their *in vivo* phenotype. It would provide a tremendous advantage to have a system that was capable of maintaining epithelial cell growth *in vitro* wherein the cells maintained their normal phenotype.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, approximately 42,000 corneal transplants were performed in the United States in 1993. Human epidermal cells can already be grown *in vitro* and used to populate burn sites and chronic skin ulcers. However, many primary cells and tissues are difficult to establish *in vitro* on normal tissue culture plastic. Although this problem is partially alleviated by the use of extracellular matrix-coated cell supports, this is only a temporary solution.

There is a need to induce hemidesmosome formation in cells normally unable to do so themselves. There is also a need to effectively maintain tissues and organs *ex vivo*. The present invention satisfies these needs.

Summary of the Invention

One aspect of the present invention provides a method for growing epithelial cells *in vitro*, comprising:

growing 804G rat bladder carcinoma cells in

media under conditions that promote the secretion of soluble factors, wherein the 804G cells secrete the factors;

removing the media from the 804G cells; and

contacting the media with epithelial cells such that the contact promotes hemidesmosome formation in the epithelial cells.

Preferably, the epithelial cells are mammalian; most preferably, they are human. According to another aspect of this preferred embodiment, the cells are human skin cells.

The present invention also provides an article of manufacture, comprising:

a biocompatible shaped article adapted for use *in vivo* in a mammal; and

a hemidesmosome formation-facilitating protein composition on the shaped article.

Preferably, the protein composition is secreted by a tumor cell line of epithelial origin; most preferably, the cell line is the rat bladder carcinoma cell line 804G or NBT-II.

Another embodiment of the present invention is a method for inducing hemidesmosome formation in epithelial cells *in vitro*, comprising the step of culturing epithelial cells unable to themselves form hemidesmosomes in the presence of an effective, hemidesmosome-formation facilitating amount of a soluble factor in aqueous solution, wherein the soluble factor is the hemidesmosome inducing soluble factor secreted by 804G rat bladder carcinoma cells.

Advantageously, the hemidesmosome inducing factor may be obtained from either 804G or NBT-II cells. Additionally, the 804G cells may be adapted to grow in low serum medium. Preferably, the epithelial cells are human. According to another aspect of this embodiment, the soluble factor is provided to the cells in an

essentially cell-free medium. Alternatively, the soluble factor is provided to the cells in a low serum medium.

Still another aspect of the invention is a method for facilitating growth of or hemidesmosome formation by epithelial cells, comprising the steps of:

providing a cell-free aqueous medium containing an effective cell growth-facilitating or hemidesmosome formation-facilitating amount of the soluble factor produced by 804G cells that facilitates hemidesmosome formation in epithelial cells; and

contacting epithelial cells that are not producing effective amounts of the soluble factor with the aqueous medium.

Preferably, the epithelial cells are human.

According to another aspect of the invention, there is provided an 804G cell line that has been adapted to grow in a low serum medium, such that the cell line secretes a soluble hemidesmosome formation-inducing factor.

Yet another embodiment of the invention is the active hemidesmosome-inducing soluble factor that is produced by the cell line 804G, in substantially isolated or purified form. Preferably, the soluble factor is provided in a pharmaceutically acceptable carrier.

The present invention also provides an article of manufacture, comprising:

a trans-epithelial appliance; and

a hemidesmosome formation-inducing composition deposited on the appliance, wherein the composition is hemidesmosome-inducing 804G soluble factor.

Preferably, the appliance is an indwelling catheter, needle, metal pin, metal rod, colostomy tube, dental abutment piece or surgical mesh. Advantageously, the composition is a soluble factor secreted by 804G cells and the article further comprises epithelial cells

deposited on said hemidesmosome formation-inducing composition. The appliance may be used *in vivo* and may be made of or coated with a biocompatible metal, preferably stainless steel or titanium. Alternatively, the appliance may be made of or coated with a ceramic material, preferably hydroxyapatite. The appliance may also be made of or coated with a polymer, preferably polyester, polyglycolic acid or a polygalactose-polyglycolic acid copolymer.

Another embodiment of the invention is a method for inducing epithelial cell attachment to a trans-epithelial appliance, comprising coating the appliance with a hemidesmosome formation-inducing composition prior to incubation with epithelial cells, wherein the composition is hemidesmosome-inducing 804G matrix protein. Preferably, the composition is a soluble factor secreted by 804G cells and the appliance is either an indwelling catheter, needle, metal pin, metal rod, colostomy tube, dental abutment piece or surgical mesh. It is preferred that the appliance is made of or coated with a polymer, preferably polyester, polyglycolic acid or a polygalactose-polyglycolic acid copolymer.

The present invention also provides a method for preserving corneal explants *ex vivo*, comprising culturing the explants in a medium containing a hemidesmosome-inducing protein factor, wherein the factor is the hemidesmosome-inducing soluble factor secreted by 804G rat bladder carcinoma cells. Preferably, the medium is 804G conditioned medium.

The present invention also provides a method for inducing epithelial cell attachment to a surface, comprising applying a hemidesmosome-inducing composition to the surface, wherein the composition is hemidesmosome-inducing 804G soluble factor.

Detailed Description of the Invention

The present invention includes the discovery that a

soluble factor secreted into the growth media by certain cell lines can stimulate cellular adhesion and hemidesmosome assembly in epithelial cells. One type of cell with this ability is the rat bladder carcinoma cell line 804G. This cell line has been described by Izumi, et al., *Cancer Res.* (1981); 41:405-409, and is maintained in permanent collection in the laboratory of inventor Jonathan C. R. Jones, from whom the cell line is readily available. This cell line is also available from Ryoichi Oyasu, Department of Pathology, Northwestern University Medical School, Chicago, Illinois. The 804G cell line is also maintained as a Budapest Treaty patent deposit by the American Type Culture Collection (ATCC), Rockville, Maryland, under accession number ATCC CRL 11555 made February 24, 1994.

Furthermore, the purification of these soluble factors is greatly facilitated by culturing the 804G cells under low serum conditions due to the virtual absence of contaminating serum proteins. Although the 804G cells were cultured in Dulbecco's Modified Eagles Medium:OPTI-MEM (1:1) in the presence of 1% fetal calf serum, the use of other media and other concentrations of fetal calf serum, preferably from about 0.1% to about 5%, is also contemplated.

Ultrastructural data have been developed demonstrating that the 804G soluble factor can induce a number of different cell types to develop mature hemidesmosomes and attach to their growth substrate. A solution can now be prepared, having factors secreted by cells such as 804G cells, that can modulate the organization of hemidesmosomal antigens in unrelated cells. This effect appears specific to hemidesmosomal elements since adhesion plaque components do not obviously change their localization in cells treated with the 804G soluble factor.

To demonstrate our new discovery, we provide

evidence that the murine 804G factor was capable of inducing assembly of "mature" hemidesmosomes in human HaCaT cells. It can be appreciated that it is uncommon to find compounds from murine cells that have such a profound affect on human tissue. In the experiments described in more detail below an increased number of hemidesmosome-like structures were found in HaCaT cells treated with 804G growth media, as compared to control experiments wherein HaCaT cells were grown on rat tail collagen. Moreover, the majority of hemidesmosome-like structures in the treated cells contacted the cell-substrate and possessed basal dense plates. The basal dense plate structures are often used as indicators of mature or formed hemidesmosomes (Krawczyk and Wilgram, (1973) *J. Ultrastruct. Res.*, **45**:93-101).

Although methods relating to production and isolation of the 804G soluble factor are disclosed, it can be appreciated that any cell that secretes compounds transmitting the ability to support cell adhesion and hemidesmosome assembly *in vitro* is within the scope of the present invention. Soluble factors from other cell types, such as the murine bladder carcinoma cell line NBT-II (ATCC CRL 1655), also appear able to induce attachment and hemidesmosome assembly *in vitro*. The NBT-II cell line is also maintained as a Budapest Treaty patent deposit by ATCC under accession number ATCC CRL 11556. It should be noted that the term "804G Factor" is used herein to generically refer to any secreted cell factor with the ability to stimulate cell attachment and hemidesmosome formation.

One major use contemplated for the active components of the soluble material is in cell growth and attachment. A substrate upon which cells are to be grown is coated with a solution comprising the soluble factor. The cells to be grown are then plated on or applied to the substrate. Such cells, including human cells *in vitro*

and *in vivo*, will grow in an organized fashion on the substrate and will form hemidesmosomes. Hemidesmosome formation is a major advantage, because it greatly enhances the attachment of the cells to the substrate. Furthermore, it appears that the organization of cells stimulated by the soluble factor is significantly more advanced and more tissue-like, than cells grown without stimulation by the active components in the secreted factors of the present invention.

The substrate used herein may be any desired substrate. For laboratory use, the substrate may be as simple as glass or plastic. For use *in vivo*, the substrate may be any biologically compatible material on which cells can grow. Suitable substrate materials may include shaped articles made of or coated with such materials as collagen; regenerated collagen; polylactic acid; biocompatible metals such as stainless steel and titanium; ceramic materials including prosthetic materials such as hydroxylapatite; synthetic polymers, including polyesters and nylons; and virtually any other material to which biological molecules can readily adhere.

One particular use of the present invention is to increase epidermal cell adhesion to target surfaces. For instance, prostheses for dental implantation such as dental abutment pieces may be coated with the 804G soluble factor to stimulate periodontal cell attachment. Existing teeth may similarly be coated as a treatment for gum (junctional epithelium) disease, such as gingivitis. Where a substrate is made of polymers of natural or synthetic bioerodible material in the form of a sheet or fabric, such as woven or bonded collagen, polylactic acid, lactide, glycolide, glutamic acid, collagen or albumin the matrix materials may be applied to the surface thereof or mixed in with the composition. Cells (such as epidermal cells) may then be grown on the matrix

ex vivo to form transplantable or implantable materials; alternatively, the materials may be implanted and cells may be permitted to attach *in vivo*.

The soluble 804G hemidesmosome-inducing factor secreted by 804G cells can also be used to coat other appliances, referred to herein as "trans-epithelial" appliances. A trans-epithelial appliance is any appliance capable of penetrating the epithelium, including but not limited to needles, metal pins or rods, indwelling catheters, colostomy tubes and surgical meshes made of biocompatible materials. The individual protein components of the matrix may also be isolated and used to coat the appliance. Alternatively, the conditioned medium from 804G cells may be used to coat the appliance. Moreover, protein components of the soluble factor may be recombinantly produced and used as an appliance coating. The coating of any desired surface capable of supporting cell adhesion with the 804G soluble factor or any protein component thereof is within the scope of the present invention.

The appliance may be immersed in, incubated in or sprayed with the conditioned medium from 804G cells grown under low or normal serum conditions. The growth of 804G cells under low serum conditions facilitates the purification of the factor from the medium as described om Example 6 hereinbelow. The purified or recombinantly produced soluble factor may also be applied to the appliance in the same manner as described hereinabove. In a preferred embodiment, the concentration of the factor used for coating the apliance is between about 20μg/l and about 200 μg/l. In a particularly preferred embodiment, the concentration is between about 50 μg/l and about 150 μg/l.

The conditioned medium may also be used to support tissue and organ growth *ex vivo*. In human tissue explant culture, 804G matrix is utilized by cells and is

incorporated into preexisting basement membranes. For example, in human corneal rims, the soluble laminin variant-containing 804G cell conditioned medium has been used for maintenance of epithelial cell attachment in corneas (Example 11) and induction of assembly of an essential epithelial cell-matrix attachemnt device in the same tissue.

The corneas may be placed directly in conditioned medium from 804G cells or may be placed in conventional medium supplemented with 804G conditioned medium. The amount of 804G conditioned medium required for optimal corneal maintenance *ex vivo* will vary depending on the confluency, passage number and particular growth conditions of the cell, although the use of between 10% and 100% conditioned medium (the remainder being normal medium) is contemplated. Optimization of the amount of conditioned medium to use may be determined by one of ordinary skill in the art using routine experimentation. The maintenance of other tissues and organs *ex vivo* in 804G conditioned medium and 804G conditioned medium-supplemented normal medium is also within the scope of the invention.

Although methods related to the production and isolation of the 804G soluble factor are specifically disclosed, it will be appreciated that any soluble factor having the ability to support cell adhesion, spreading and hemidesmosome formation is within the scope of the present invention.

The 804G soluble factor will also be of great use in studies concerning hemidesmosome morphogenesis and $\alpha_6\beta_4$ integrin interactions with the epithelial extracellular matrix. Indeed, the active factors secreted by the 804G cells may prove to be a tool that allows definition of hemidesmosome-mediated interactions between epithelial cells and their underlying connective tissues at the molecular level.

In addition to the soluble factor and the active components thereof, the present invention also includes shaped articles coated with those materials. Preferably, those shaped articles are formed of materials other than glass, and include such forms as sheets, fabrics, prostheses, metal articles, bioerodible articles, and implantable articles.

Furthermore, pharmaceutical preparations having the soluble factor are contemplated. These preparations can be in any suitable form, and generally comprise the active ingredient in combination with any of the well known pharmaceutically acceptable carriers. The soluble factor may be isolated from the growth media in which appropriate cells have been grown. Alternatively, the soluble factor may be prepared synthetically or through recombinant DNA techniques, or through purification of isolated proteins from the growth media.

Carriers can include injectable carriers, topical carriers, transdermal carriers, and the like. The preparation may advantageously be in a form for topical administration, such as an ointment, gel, cream, spray, dispersion, suspension, or paste. The preparations may further advantageously include preservatives, antibacterials, antifungals, antioxidants, osmotic agents, and similar materials in composition and quantity as is conventional. For assistance in formulating the compositions of the present invention, one may refer to Remington's Pharmaceutical Sciences, 15th Ed., Mack Publishing Co., Easton PA (1975).

Finally, epithelial cells of various types may be grown in contact by the compositions contemplated herein.

As a first step in discovering the properties of the 804G soluble factor, HaCaT cells were treated with media from growing 804G cells.

Example 1

Soluble Factor Treatment of HaCaT Cells

The immortalized human keratinocyte cell line HaCaT, provided by Dr. Norbert Fusenig, Heidelberg, Germany (Boukamp, et al. (1988) *J. Cell Biol.*, **106**:761-771), was cultured in DMEM medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum (FCS, Bio-Whittaker) and antibiotics. The HaCaT cell line has normal keratinization properties *in vitro*, is positive for involucrin, filaggrin, cytokeratins 1, 10, 5, 6, 14, 16/17, 7, 8 and 19 and is negative for vimentin. Thus it has characteristics very similar to primary keratinocytes.

The rat bladder carcinoma cell line, 804G, and the human embryonic fibroblast cell line WI-38 (ATCC CCL 75) were also cultured in DMEM medium with the same supplements.

Culture supernatant of 804G cells was collected from cultures that were approximately 70% confluent and reached confluence over a 48 hour period. At the end of this time 15 mls of supernatant was collected from a 75 cm^2 culture flask. Supernatants from HaCaT and WI-38 cells were collected in the same manner over a 48 hour period.

HaCaT cells plated on tissue culture plastic in normal medium attach, spread very slowly, and still appear rounded 2 hours after seeding. In contrast, when the cells were seeded in the culture supernatant of 804G cells they attached to the growth substratum and acquired a flattened morphology within 30 minutes. After 24 hours, cells in normal medium formed epithelioid islands whereas cells seeded in supernatant from 804G cells exhibited a spread-out morphology and appeared to migrate so as to uniformly cover the growth substratum. The 804G culture supernatant effect was evident even if the cells were plated in a 1:1 dilution of the supernatant with normal medium. As a control, HaCaT cells were also plated in their own culture supernatant and in medium

collected from cultures of human fibroblasts (WI-38). HaCaT cells plated in either their own medium or WI-38 medium did not exhibit the growth and morphology of those cells plated in 804G medium.

We then performed the following experiments to analyze the affect of the 804G supernatant on hemidesmosome elements in the HaCaT cells.

Example 2

Analysis of Hemidesmosome Development After Treatment with the 804G Supernatant

To study the effect of 804G culture supernatants on the distribution of $\alpha_6\beta_4$ integrins, HaCaT cells were grown on glass coverslips, fixed and immunolabeled for α_6 and β_4 integrin subunits, hemidesmosomal components, or epithelial matrix elements.

HaCaT cells were grown for 24 hours on glass coverslips for immunofluorescence microscopy either in normal medium, medium conditioned for 48 hours with 804G cells, or in co-culture (1:1) with 804G cells. The cells were fixed for 5 minutes in -20°C methanol, washed in PBS and immunolabeled with the following antibodies:

(1) AA3; a mouse monoclonal antibody to the human β_4 integrin subunit (Tamura, et al., J. Cell Biol. 1990; 111:1593-1604). This antibody specifically binds to human integrin molecules.

(2) GOH3; a rat monoclonal antibody to the α_6 integrin subunit (AMAC, Westbrook, ME). This antibody reacts with human and mouse, but not rat integrin molecules.

(3) 6844; a rabbit polyclonal antiserum to the cytoplasmic terminal 15 amino acids of the α_6 integrin subunit.

(4) J18; a rabbit antiserum to the solubilized matrix of 804G cells (Langhofer, et al., (1993) *J. Cell Sci.*, **105**:753-764).

(5) 5C5; a mouse monoclonal antibody the solubilized

matrix of 804G cells.

(6) J17; a rabbit antiserum against the 180 kD hemidesmosomal protein (Riddelle, et al., (1992) *J. Cell Sci.*, **103**:475-490).

(7) P1E1; a mouse monoclonal antibody to epiligrin (from Dr. William G. Carter, Fred Hutchinson Cancer Research Center, Seattle, WA, Carter et al., 1991).

(8) BM165; a mouse monoclonal antibody to kalinin (from Dr. Robert E. Burgeson, Oregon Health Sciences University, Portland, OR, Rousselle, et al., 1991, Marinkovich, et al. 1992).

(9) GB3; a mouse monoclonal antibody to human basement membranes (Accurate Chemical and Scientific Corporation, Westbury, NY).

Fluorescein isothiocyanate (FITC) or tetramethyl-rhodamine isothiocyanate (TRITC) conjugated anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). FITC or TRITC conjugated anti-rat antibodies were from Sigma Chemical Corporation (St. Louis, MO).

After single or double immunolabeling the cells were studied under a Zeiss Axlophot microscope equipped with epifluorescence and phase contrast optics. Photographs were taken with a Leitz Orthomat E automatic camera system and Kodak TMY 400 film at EI 800.

The visualization and photography of living cells was performed with a Zeiss Axiovert microscope equipped with phase contrast optics and the same camera system as above.

In cells grown in normal medium, the α_6 and β_4 integrin subunits had a patchy, finely granular distribution most clearly visualized at the edges of cell islands. In cells grown in the 804G supernatant, the α_6 and β_4 subunits were reorganized into coarsely granular or "Swiss-cheese" type patterns. The same patterns were reflected in immunolabeling with any of the antibodies

against epithelial matrix elements (P1E1, GB3, BM165) and with the antibody against the 180 kD hemidesmosomal protein (J17).

Example 3

Electrophoretic Analysis of the Culture Medium

Polypeptide samples from the culture medium and solubilized matrix from 804G and HaCaT cells were analyzed by SDS-polyacrylamide gel electrophoresis on a 6% resolving gel (Laemmli 1974) with the NOVEX (Encinitas, CA) electrophoresis system. The separated polypeptides of culture medium, or purified rat laminin and fibronectin (as controls, Telios Pharmaceuticals/GIBCO, Grand Island, NY) were electrophoretically transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA) and processed for immunoblotting with J18 and 5C5 antibodies. As controls, the polypeptides were immunoblotted with rabbit antiserum against rat laminin or rat fibronectin (Telios Pharmaceuticals, San Diego, CA). The Vectastain ABC immunoperoxidase or alkaline phosphatase were used to detect binding. (Vector Laboratories, Burlingame, CA).

The polyclonal antibody, J18 reacted with these polypeptides in immunoblotting experiments. Immunoblotting revealed further that the antibody did not cross-react with laminin or fibronectin, two common extracellular matrix molecules. However, the 804G cell matrix and medium do contain fibronectin, but only trace amounts of laminin-related material.

The monoclonal antibody 5C5 also reacts with the major polypeptides in 804G matrix. Immunoblotting of the HaCat matrix reveals the same three 160-138 kD polypeptides as in the 804G matrix with an additional polypeptide having a molecular weight of approximately 130 kD. The 5C5 antibody is specific to rat proteins and therefore does not react with the HaCaT cell matrix grown in normal conditions.

In the culture medium of 804G cells the Mr 160-138 polypeptides are not clearly discernible by protein staining but can, instead, be identified by fluorography of metabolically ^{35}S-labeled proteins.

Example 4

Metabolic Labeling of 804G Cells

Metabolic labeling with ^{35}S-methionine was performed by first incubating the cells for 30 minutes in starving medium (MEM-medium without methionine, supplemented with 1% dialyzed FCS, L-glutamine and antibiotics, GIBCO, Grand Island, NY), and then replacing the medium with 3 ml of fresh MEM without methionine, supplemented with 1% dialyzed FCS and 250 μCi ^{35}S-methionine (Trans-Label ICN Biomedicals Inc., Costa Mesa, CA) for 10 hours.

Gels with radioactively labeled polypeptides were processed for fluorography according to Bonner and Laskey (1974) and exposed to Hyperfilm (Amersham Corporation, Arlington Heights, IL).

The incorporation of ^{35}S-methionine indicated that the polypeptides were synthesized by the cells and not simply deposited into the matrix from the culture medium. Moreover, the polypeptides can be visualized by immunoblotting with J18 and 5C5 antibodies. When HaCaT cells are grown in 804G cell supernatant, and their matrix is processed for immunoblotting with 5C5, two reactive polypeptides can be identified indicating that the soluble immunoreactive material from 804G supernatant is able to bind to the matrix of HaCaT cells.

The effect of the soluble 804G cell components on HaCaT cells was also evident at the ultrastructural level. When HaCaT cells are grown on cell culture plastic in normal culture medium, they attach to their growth substratum by means of extracellular matrix contacts that resemble rudimentary hemidesmosomes. Alternatively, when the cells were grown in 804G cell supernatant, they formed adhesion complexes that are by morphological criteria mature hemidesmosomes.

Example 5

Immunodepletion of 804G Supernatant with J18 Antibodies

When the cells were grown in 804G supernatant

immunodepleted with J18 antibody, only occasional hemidesmosomes can be identified at the ventral plasma membrane. Immunodepletion of 804G supernatant was carried out by treating 1 ml of supernatant three consecutive times with the J18 antibody coupled to 50 µl protein A Sepharose beads (2 µg antiserum/10 µl packed beads, Sigma Chemical Corporation). As a control, 804G supernatant was also depleted with normal rabbit serum coupled to protein A Sepharose. The cells were fixed in modified Karnowsky fixative (1% paraformaldehyde, 0.1 M Na-cacodylate, 1.75% glutaraldehyde, 2.5 mM CaCl) and processed for electron microscopy by routine methods. Thin sections were cut perpendicular to the cell layer and studied at 75 kV in a Hitachi Hu-12A microscope. The specificity of this effect is verified by the fact that immunodepletion with normal rabbit serum does not affect the hemidesmosome inducing potential of 804G supernatant.

To facilitate purification of secreted proteins, the 804G cell line was adapted to grow under low serum conditions as described in the following example.

Example 6

Growth of 804G cells under low serum conditions

804G cells were gradually adapted to grow in 1:1 DMEM:OPTI-MEM (GIBCO, Grand Island, NY) supplemented with 1% FCS, 2 mM glutamine, 100 µg/ml penicillin and 50 µg/ml streptomycin. The resulting 804G cell subpopulation was named 804GMH. According to the manufacturer, OPTI-MEM contains low amounts of transferrin and insulin, molecular weights 80 and 6 kDa, respectively, but no other proteins.

The virtual absence of serum proteins in the culture medium simplifies the purification of hemidesmosome-inducing soluble factors as described below.

Example 7

Purification of soluble factors from 804GMH culture medium

For the collection of serum-free culture supernatant, confluent 804GMH cells grown under low serum conditions were removed by trypsinization (0.02%), washed once with DMEM containing 10% FCS and cultured in DMEM:OPTI-MEM with no added FCS at a split ratio of 1:6. Culture supernatant was collected when 804GMH cells had been confluent for 24 hours. The supernatant was centrifuged at 5,000 x g for 10 minu and stored at -20°C until use. Secreted proteins were purified by precipitation with ammonium sulfate at 40% saturation. Culture supernatant (1 liter) was cleared of particulate material by centrifugation at 10,000 x g for 30 minutes and transferred to another container on ice. Ammonium sulfate was slowly added, with stirring, to 30% saturation. The supernatant was then left at 4°C overnight to allow complete precipitation. The sample was centrifuged for 30 min at 10,000 x g and ammonium sulfate added to a final concentration of 40% saturation. After precipitation and centrifugation, the supernatant was discarded and the pellet resuspended in 1 ml PBS. The protein was dialyzed against PBS, the protein concentration estimated by absorbance at 280 nm, and an aliquot analyzed by SDS-PAGE. Bands of 240, 150 and 140 kDa were observed.

Thus, we have demonstrated that soluble factors produced by 804G cells are able to induce attachment and hemidesmosome assembly in mammalian cells and that the purification of these proteins from the culture medium is greatly facilitated by growing the cells under low serum conditions.

Example 8

Adhesion of Epithelial Cells to Soluble Factor-Coated Dental Implants

The three types of titanium implants used were: IMZ titanium plasma sprayed (Interpore International, Irvine, CA), HA-coated titanium implant (Calcitek, Carlsbad, CA),

and a screw-vent titanium implant (Dentsply, Inc., Encino, CA). The implant from Interpore had a polished titanium collar that was not covered with the sprayed titanium and the Calcitek implant came with a polished titanium healing screw.

The implants were thoroughly cleaned with a detergent solution, extensively rinsed with tap water followed by deionized water and allowed to dry. Implants were sterilized by immersion in 95% ethanol, rinsed in sterile PBS lacking calcium and magnesium (Bio-Whittaker) and air-dried in a sterile petri dish.

One sample of each type of implant was left untreated, one was coated with 804G culture medium (DMEMC=DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin), and one was coated with 804G conditioned medium collected after four days of cell growth. Coating was performed by placing the implants into sterile 0.65 ml tubes containing DMEMC, 100 μl 804G conditioned medium, or nothing (untreated control). The implants were placed into the solutions upside down to ensure coating of the exposed polished titanium on the Interpore and Calcitek implants. The samples were then placed at 4°C overnight (about 16 hours). The implants were removed form the coating solutions and placed into six well tissue culture plates, one implant per well. Nonspecific binding sites on each implant were blocked with 5 ml of 1% (w/v) bovine serum albumin (BSA) in PBS for 5 hours at room temperature. The blocking solution was removed and the implants were washed three times with PBS.

FGmet2 human pancreatic carcinoma cells, an epithelial cell line, were used to test for rapid cell adhesion to the coated implants. The cells were trypsinized and centrifuged at 1500 rpm for 5 minutes. The cell pellet was washed twice by resuspension in 1%

BSA in DMEM and centrifuged. The cell pellet was resuspended in 1% BSA in DMEM to a final concentration of 2.2×10^6 cells/ml. The six well plates were tilted to allow the implants to rest against one edge of the well and the implants were overlayed with 1 ml of the cell suspension. The cells were incubated with the implants for 30 min at 37°C, removed by aspiration, and the implants washed three times with PBS. The cells were fixed for 5 minutes with 3% paraformaldehyde in 2% sucrose and PBS, and stained for 15 minutes with 0.5% crystal violet in 20% methanol. The excess dye was removed by rinsing under tap water and the implants were examined using an inverted phase microscope.

Significant FGmet2 cell attachment and spreading was observed only on the implants coated with the 804G conditioned medium. This result indicates that hemidesmosome formation-inducing factors secreted by 804G cells can induce epithelial cell attachment and spreading on a shaped, trans-epithelial appliance.

The ability of 804G matrix to coat absorbable and nonabsorbable surgical meshes and the subsequent ability of the matrix to support rapid adhesion and cell proliferation was assessed as described in the following two examples.

Example 9

Rapid Adhesion of Epithelial Cells to a Surgical Mesh

804G conditioned medium was used as a source of soluble matrix protein. A small piece of polypropylene (PROLENE™), polyester (MERSILENE™), and polyglactin (Vicryl™, a biodegradable copolymer comprising 90% glycolide, a polyglycolic acid derivative and 10% glactide, a polygalactose derivative) mesh (all from Ethicon, Inc.) were each placed into wells of a 24 well tissue culture plate containing either 1 ml 804G conditioned medium or 1 ml DMEM complete medium and incubated overnight at 4°C. The meshes were washed twice

with PBS containing 1% BSA (PBS + BSA) and nonspecific binding sites were blocked with PBS + BSA for one hour at room temperature. 4 x 10^5 FGmet2 cells in 1 ml DMEM + 1% BSA + 25 mM HEPES were pipetted on top of the meshes and allowed to incubate at 37°C for 35 min. The meshes were then transferred into a 6 well tissue culture plate and washed three times for 5 min each in 5 ml PBS. The meshes were fixed in 1 ml 3% paraformaldehyde + 2% sucrose in PBS for 5 min at room temperature and the adherent cells stained with 0.5% crystal violet in 20% methanol for 15 min at room temperature. The meshes were washed extensively with water to remove nonspecific staining.

The results indicated that both the 804G-treated Mersilene™ and Vicryl™ meshes visibly stained darker than the control-treated meshes. Thus, the polyester and polyglactin 910 meshes supported 804G matrix adhesion and, more importantly, promoted rapid adhesion of epithelial cells to these materials. In contrast, no detectable cell staining was observed with the 804G-treated Prolene™ mesh which is consistent with the observation that polypropylene has a low capacity for binding proteins.

Example 10

Growth of Epithelial Cells on 804G Matrix-Precoated Surgical Meshes

Mersilene™ and Vicryl™ meshes were precoated in 1 ml degassed 804G conditioned medium or degassed DMEM complete media containing 25 mM HEPES overnight at 4°C. Both mediums were degassed for 30 min at room temperature with a vane pump drawing a 23 mm Hg vacuum. The meshes were washed twice with sterile PBS and 1 ml RPMI complete medium containing 8 x 10^4 FGmet2 epithelial cells was pipetted on top of the meshes and allowed to incubate at 37°C.

After one day of growth, FGmet2 cells were visibly attached and spreading on 804G-treated meshes. The loose weave of the Mersilene™ mesh permitted better visualization of the cells than the tight weave of the Vicryl™ mesh. After two days the meshes were transferred to a new plate, fresh medium was added and the incubation was continued. After five days, cells were growing extensively along the Mersilene™ mesh fibers and appeared to cover more than 50% of the fiber surface. In contrast, cells growing on the control-treated mesh grew into a ball-shaped structure and did not exhibit significant growth along the fiber surface. These results demonstrate the unique ability of the soluble 804G matrix to adsorb onto medically important surfaces and promote the attachment and proliferation of cells on these materials.

Example 11

Preservation of Corneal Explants with 804G Soluble Factor

Human donor corneal rims procured following penetrating keratoplasties were maintained in DMEM containing FCS (DMEM-) or in the same medium supplemented with soluble factors, including adhesion complex-associated matrix components, that are secreted in large amounts by 804G cells (DMEM+). After 72 hours, the

tissue was processed for electron and immunofluorescence microscopy using various adhesion complex antibodies.

The epithelial layers became detached from the underlying stroma in corneal rims maintained in DMEM-. This detachment was correlated with a loss of adhesion complexes and their protein constituents. In contrast, after 72 hours in DMEM+, the epithelial layers appear healthy with numerous adhesion complexes in regions of cell-stromal attachment. In this wound model, no morphologic hemidesmosomes were observed in epithelial cells repopulating "wounds" in tissue material maintained in DMEM-. However, in DMEM+ media, morphologic hemidesmosomes were seen along the bare stroma in areas of epithelial cell-wound bed interaction.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

It should be noted that the present invention is not limited to only those embodiments described in the Detailed Description. Any embodiment which retains the spirit of the present invention should be considered to be within its scope. However, the invention is only limited by the scope of the following claims.

WE CLAIM:

1. An article of manufacture, comprising:

a biocompatible shaped article adapted for use *in vivo* in a mammal; and

a hemidesmosome formation-facilitating soluble factor deposited on said shaped article.

2. The article of Claim 1, wherein said soluble factor is a type that is secreted by a tumor cell line of epithelial origin.

3. The article of Claim 2, wherein said tumor cell line is the rat carcinoma cell line 804G.

4. The article of Claim 2, wherein said tumor cell line is the rat bladder cancer cell line NBT-II.

5. The article of Claim 1, wherein said shaped article is a trans-epithelial appliance.

6. The article of Claim 5, wherein said trans-epithelial appliance is selected from the group consisting of indwelling catheter, needle, metal pin, metal rod, colostomy tube, dental abutment piece and surgical mesh.

7. The article of Claim 1, further comprising epithelial cells deposited on said hemidesmosome formation-inducing soluble factor.

8. The article of Claim 5, wherein said appliance is used *in vivo*.

9. The article of Claim 5, wherein said appliance is made of or coated with a biocompatible metal.

10. The article of Claim 9, wherein said metal is stainless steel or titanium.

11. The article of Claim 5, wherein said appliance is made of or coated with a ceramic material.

12. The article of Claim 11, wherein said material is hydroxyapatite.

13. The article of Claim 5, wherein said appliance is made of or coated with a polymer.

14. The article of Claim 13, wherein said polymer

is selected from the group consisting of polyester, polyglycolic acid and a polygalactose-polyglycolic acid copolymer.

15. A method for inducing growth of or hemidesmosome formation by epithelial cells *in vitro*, comprising the step of:

culturing epithelial cells unable to themselves form hemidesmosomes in the presence of an effective, hemidesmosome-formation facilitating amount of a soluble factor in aqueous solution, wherein said soluble factor is the hemidesmosome inducing soluble factor that is secreted by 804G rat bladder carcinoma cells.

16. The method of Claim 15, wherein said hemidesmosome inducing factor has been obtained from NBT-II rat bladder carcinoma cells.

17. The method of Claim 15, wherein said hemidesmosome inducing factor has been obtained from 804G cells.

18. The method of Claim 17, wherein said 804G cells have been adapted to grow in a low serum medium.

19. The method of Claim 15, wherein said epithelial cells are human.

20. The method of Claim 15, wherein said soluble factor is provided to said cells in a medium essentially free of cells.

21. The method of Claim 15, wherein said soluble factor is provided to said cells in a low serum medium.

22. An 804G cell line that has been adapted to grow in a low serum medium, wherein said cell line secretes a soluble hemidesmosome formation-inducing factor.

23. The active hemidesmosome-inducing soluble factor that is produced by the cell line 804G, in substantially isolated or purified form.

24. The soluble factor of Claim 23, in a

pharmaceutically-acceptable carrier.

25. A method for inducing epithelial cell attachment to a trans-epithelial appliance, comprising coating said appliance with a hemidesmosome formation-inducing composition prior to incubation with epithelial cells, wherein said composition is hemidesmosome-inducing 804G soluble factor.

26. The method of claim 25, wherein said composition is a soluble factor secreted by said 804G cells.

27. The method of Claim 25, wherein said appliance is selected from the group consisting of indwelling catheter, needle, metal pin, metal rod, colostomy tube, dental abutment piece and surgical mesh.

28. The method of Claim 25, wherein said appliance is made of or coated with a polymer.

29. The method of Claim 28, wherein said polymer is selected from the group consisting of polyester, polyglycolic acid and a polygalactose-polyglycolic acid copolymer.

30. A method for preserving corneal explants *ex vivo*, comprising culturing said explants in a medium containing a hemidesmosome-inducing protein factor, wherein the factor is the hemidesmosome-inducing soluble factor secreted by 804G rat bladder carcinoma cells.

31. The method of Claim 30, wherein said medium is 804G conditioned medium.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61L 27/00, 29/00, 31/00 — A3

(11) International Publication Number: **WO 95/13103**

(43) International Publication Date: 18 May 1995 (18.05.95)

(21) International Application Number: PCT/US94/12894

(22) International Filing Date: 9 November 1994 (09.11.94)

(30) Priority Data:
08/151,134 12 November 1993 (12.11.93) US

(71) Applicant: DESMOS, INC. [US/US]; Building 2, Room 214, 3550 General Atomics Court, San Diego, CA 92121 (US).

(72) Inventors: QUARANTA, Vito; 8861 Nottingham Place, La Jolla, CA 92037 (US). HORMIA, Marketta; 3665 Morlan, San Diego, CA 92117 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson & Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
6 July 1995 (06.07.95)

(54) Title: SOLUBLE FACTOR STIMULATION OF ATTACHMENT AND HEMIDESMOSOME ASSEMBLY IN EPITHELIAL CELLS

(57) Abstract

A method for growing epithelial cells *in vitro* using soluble proteins secreted by 804G rat bladder carcinoma cells. These proteins are able to stimulate cell attachment and hemidesmosome formation in cells grown in contact with the proteins. The purification of these proteins from 804G culture supernatant is greatly facilitated by culturing the cells under low serum conditions. Trans-epithelial appliances coated with the soluble proteins and the use of the soluble proteins in the maintenance of human tissue *ex vivo* are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 94/12894

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 A61L29/00 A61L31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 03392 (REGENTS OF THE UNIVERSITY OF MINNESOTA) 20 April 1989 see page 18, line 17 - line 36 ---	1,2,5-14
P,X	WO,A,94 23016 (DESMOS,INC.) 13 October 1994 see the whole document ---	1-31
A	JOURNAL OF CELL SCIENCE, vol. 105, 1993 GB, pages 753-764, MIKAYO LANGHOFER ET AL. 'THE MATRIX SECRETED BY 804G CELLS CONTAINS LAMININ-RELATED COMPONENTS THAT PARTICIPATE IN HEMIDESMOSOME ASSEMBLY IN VITRO.' see abstract --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 May 1995	09.06.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF CELL BIOLOGY, vol. 112,no. 1, January 1991 US, pages 159-168, K.S. RIDDELLE ET AL. 'FORMATION OF HEMIDESMOSOMES IN VITRO BY A TRANSFORMED RAT BLADDER CELL LINE' see the whole document ---	1-31
A	CELL REGULATION, vol. 2, June 1991 US, pages 427-438, J.C.R. JONES ET AL. 'A FUNCTION FOR THE INTEGRIN A6B4 IN THE HEMIDESMOSOME.' see abstract ---	1-31
A	JOURNAL OF CELL SCIENCE, vol. 103,no. 2, October 1992 GB, pages 475-490, K.S. RIDDELLE ETAL. 'HEMIDESMOSOMES IN THE EPITELIAL CELL LINE 804G: THEIR FATE DURING WOUND CLOSURE, MITOSIS AND DRUG INDUCED REORGANIZATION OF THE CYTOSKELETON' see abstract -----	1-31

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/US 94/12894

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8903392	20-04-89	US-A-	4876332	24-10-89
		DE-A-	3880318	19-05-93
		EP-A,B	0380546	08-08-90
		JP-T-	3500492	07-02-91
		US-A-	5007925	16-04-91
		US-A-	5242826	07-09-93
WO-A-9423016	13-10-94	AU-B-	6555794	24-10-94